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Development of an *in situ* Hybridization Histochemistry for Choline Acetyltransferase mRNA with RNA Probes

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ABSTRACT—An *in situ* hybridization histochemistry (ISHH) for choline acetyltransferase (ChAT) in paraffin sections of the central nervous system of the rat was developed using ^{35}S-labeled RNA probes by defining the technical parameters enabling optimal detection of ChAT mRNA. Fixation by perfusion with 4% paraformaldehyde and 0.4% glutaraldehyde provided the most intense signals. Slides for mounting paraffin sections were coated with 1% BSA and fixed with 25% glutaraldehyde. Proteinase K treatment of tissue sections after fixation increased intensity of signals. Decreasing probe length increased intensity of signals; truncation to approximately 75 nucleotides by alkaline hydrolysis provided the best result. Ribonuclease A treatment after hybridization appeared to be essential to reduce nonspecific background signals. Specificity of hybridization signals was confirmed by the selective localization of signals in motoneurons in the spinal cord with an antisense probe and no signal in them with a sense probe. Distribution patterns of perikarya of neurons in the forebrain, cranial nerve motor nuclei and cervical region of the spinal cord revealed by ISHH and immunohistochemistry (IHC) were almost superimposable, giving additional evidence for specificity of the present method. However, intensity of signals for ChAT mRNA detected by ISHH and cellular content of ChAT protein revealed by IHC were not always parallel.

INTRODUCTION

Considerable progress has been made in elucidating the organization of the central cholinergic system by immunohistochemistry (IHC) using monoclonal antibodies to choline acetyltransferase (ChAT), the most specific marker for cholinergic neurons [1]. Compared to the abundance of information on cholinergic neurons in the adult brain, information on the developing cholinergic system is limited [2–5]. This may be due in part to the lack of monoclonal antibodies applicable to cryostat or paraffin sections and difficulties of working with free-floating sections of delicate embryonic and early postnatal brain tissues. By introduction of molecular biological techniques, two methods are available to overcome the disadvantage of the monoclonal antibody.

One method involves the expression of protein from a cloned gene introduced into *Escherichia coli* and the production of antisera against the protein. We have succeeded in expressing large amounts of rat ChAT protein using rat ChAT cDNA ligated to a translation vector, and in producing an antiserum. This antiserum is highly specific to rat ChAT, immunochemically and immunohistochemically, and stains not only perikarya and dendrites but also axons and terminals of cholinergic neurons in cryostat sections [6].

The second method involves an *in situ* hybridization histochemistry (ISHH), which enables the precise localization and identification of individual cells in which a particular gene is transcribed. Among probes in current use, RNA probes offer a unique combination of advantages for ISHH: (1) antisense probes contain only the antisense strand and no sense strand to compete in solution for hybridization with target mRNAs, resulting in much higher signals, (2) control probes are easily prepared, (3) fragment length of probes can be reproducibly controlled by limited alkaline hydrolysis [7], providing good penetration of probes into tissue, (4) RNA-RNA hybrids have higher stability compared to DNA-RNA hybrids, allow-

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ing use of more stringent washing conditions to reduce nonspecific binding of probes to tissue, and (5) nonspecific background signals, in the form of probe sticking to tissue, can be reduced by post-hybridization digestion with ribonuclease (RNase) [7, 8].

In the present study, we have defined the technical parameters and established procedure for ISHH for rat ChAT mRNA in paraffin sections with RNA probes. Further, distribution and way of labeling of perikarya of neurons in the forebrain, cranial nerve motor nuclei and cervical region of the spinal cord of the rat revealed by ISHH and IHC were compared. A preliminary study of ISHH for rat ChAT mRNA with RNA probes has been reported [9].

MATERIALS AND METHODS

Preparation of RNA probes

The cDNA encoding rat ChAT [9] was ligated into *Eco*RI site of plasmid Bluescript (Stratagene). Orientation was confirmed by restriction mapping. After linearization with restriction endonucleases, templates were treated with proteinase K (Boehringer-Mannheim) and extracted with phenol/chloroform/isoamyl alcohol (25:24:1) (Phenol/CIA). To produce antisense and sense RNA probes, they were transcribed by T7 and T3 RNA polymerases (Stratagene), respectively, using an [α-^{35}S]UTP (400 Ci/mmole, Du Pont-New England Nuclear) to a specific activity of $4–5\times10^7$ dpm/μg according to the manufacturer's protocol. Templates were removed with deoxyribonuclease I (Promega), and RNA transcripts were extracted with Phenol/CIA and precipitated with ethanol in the presence of ammonium acetate twice. Both probes were truncated to approximately 75, 150 or 300 nucleotides by limited alkaline hydrolysis [7]. Mass average size of the probe was checked by electrophoresis on a 2% agarose gel containing formaldehyde followed by autoradiography [10].

Tissure preparation

Sixteen male Sprague-Dawley rats (200–300 g) were used. Two rats were decapitated without anesthesia and brains and spinal cords were rapidly dissected out, sliced at about 3 mm in thickness and immersed overnight in 4% paraformaldehyde (PA) in 0.1 M sodium phosphate (pH 7.4) (PB) at 4°C. On the next day, tissues were transferred to 70% ethanol and kept overnight at 4°C. Other rats were deeply anesthetized with sodium pentobarbital and perfused through the aortic cone with 0.1 M PB containing 0.9% NaCl at room temperature and at a flow rate of 20 ml/min for 4–6 min, followed by either 4% PA in 0.1 M PB (2 rats) or 4% PA and 0.4% glutaraldehyde (GA) in 0.1 M PB (12 rats) at 4°C and at the same flow rate for 8–12 min. Brains and spinal cords were dissected out, sliced at about 3 mm in thickness, postfixed in the same fixative at 4°C for 2 hr and kept overnight in 70% ethanol at 4°C. Tissues were dehydrated through graded ethanol series and embedded in paraffin (melting point 51–53°C, Merck). Sections were cut serially at 10 μm and mounted on the coated slides described below. An appropriate section was hybridized with the antisense probe and the next section with the sense probe.

Slide preparation

Clean slides were dipped in 1% BSA for 5 min and dried at 60°C. Then, they were fixed with 25% GA for 3 min, washed in autoclaved H_2O twice and dried at 60°C. They were used within 2 weeks.

In situ *hybridization*

Deparaffinized sections were either treated with proteinase K (1 and 100 μg/ml in 0.1 M Tris-HCl, pH 7.5, containing 50 mM EDTA, at 37°C for 30 min and at room temperature for 10 min, respectively) or only washed in 0.1 M Tris-HCl (pH 7.5), and dehydrated. Some sections treated with or without proteinase K were further subjected to acetic anhydride treatment [8], and dehydrated. ^{35}S-labeled probes were diluted to 1, 2 or 4×10^5 dpm/μl in a solution containing 20 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 50% freshly deionized formamide, 0.5 mg/ml *E. coli* tRNA, 10 mM dithiothreitol, 2.5 mM EDTA, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 10% dextran sulfate, and pipetted directly onto tissue sections (5 μl/cm^2). Sections were coverslipped and incubated at 50°C for 16 hr in a moist chamber. Hybridized slides were then washed in 2×

SSC (1×SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 50% formamide and 0.1% β-mercaptoethanol (BME) at 50°C for 1 hr, treated with RNase A (20 μg/ml in 10 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, Boehringer-Mannheim) at 37°C for 30 min, rinsed sequentially in 2×SSC containing 50% formamide and 0.1% BME at 50°C for 1 hr, in 1×SSC containing 50% formamide and 0.1% BME at 50°C for 2 hr and finally in 1×SSC containing 50% formamide at room temperature for 15 min, and dehydrated. Some slides were not treated with RNase A. Slides were dipped in NR-M2 emulsion (Konica), exposed at 4°C for 7–14 days and developed in Konicadol X. Appropriate sections were counterstained with hematoxylin.

Immunohistochemistry

Three male Sprague-Dawley rats (200–300 g) were used. Procedure for the tissue preparation was identical to that described earlier [11]. Brains and spinal cords were sectioned on a freezing microtome at 50 μm in the transverse plane. The monoclonal antibody to rat ChAT has been characterized in detail elsewhere [12, 13]. Procedure for IHC using ABC Kit (Vector) was identical to those described previously [11, 14].

RESULTS

Specificity of hybridization signals

The antisense RNA probe revealed ChAT transcripts exclusively in motoneurons in the medial and lateral nuclei in the lamina IX of the cervical region of the spinal cord of the rat (Fig. 1A, C). No signal was observed in the section hybridized with the sense probe (Fig. 1B). Signals for ChAT mRNA were mostly restricted to perikarya of motoneurons (Fig. 1C). These results indicate that the present ISHH is highly specific.

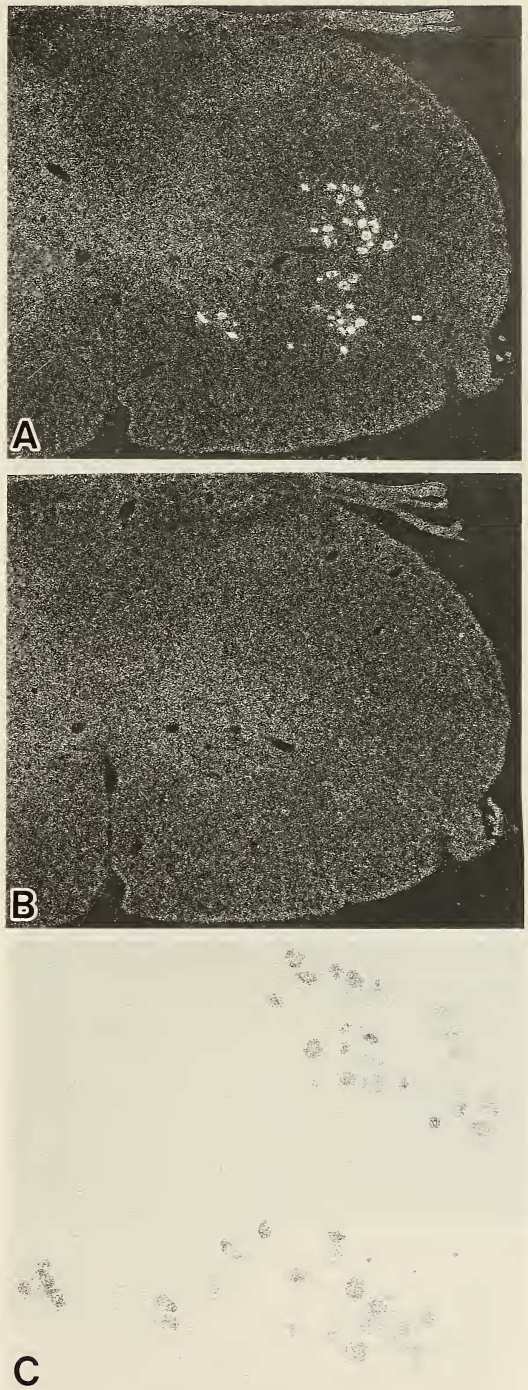

FIG. 1. ISHH of the cervical region of the spinal cord (11 days exposure). The tissue was fixed with 4% PA and 0.4% GA followed by proteinase K treatment. Probes were truncated to 75 nucleotides and applied at a concentration of 2×10^5 dpm/μl. A and B. Dark-field photomicrographs of adjacent 10-μm paraffin sections hybridized with the antisense (A) and sense (B) probes (×30). C. Bright-field photomicrograph of motoneurons in the section hybridized with the antisense probe. No counterstaining (×80).

Technical considerations

The results obtained with the antisense probe in sections of the cervical spinal cord demonstrated the critical influence of the fixation procedure on intensity of signals (Fig. 2). The highest signals for ChAT mRNA were obtained with the tissue fixed by perfusion with 4% PA and 0.4% GA followed by proteinase K treatment (Fig. 2A). Under these conditions, background signals were very low. Weaker signals were also observed in the tissue fixed by perfusion with 4% PA without proteinase K treatment (Fig. 2B), but no signal was observed when the tissue was fixed by immersion in 4% PA with or without proteinase K treatment (Fig. 2C). Treatment of proteinase K at a concentration of 100 μg/ml at room temperature for 10 min was better than that of 1 μg/ml at 37°C for 30 min. When the fixative containing GA was used, proteinase K treatment appeared to be essential, but treatment of the enzyme to the tissue fixed only with 4% PA decreased intensity of signals significantly. Treatment with acetic anhydride did not reduce background signals.

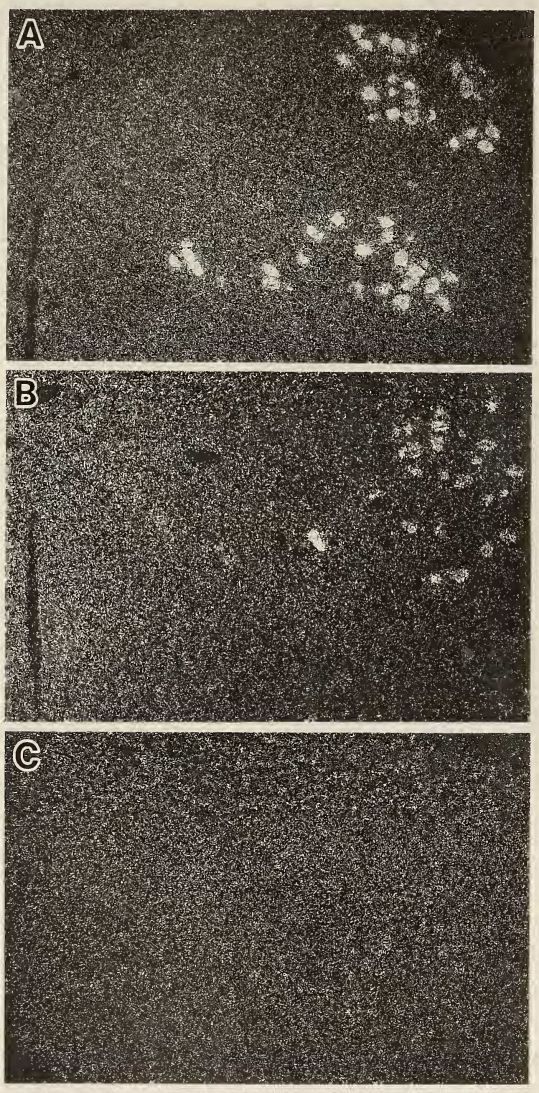

FIG. 2. Effects of fixation procedure on ISHH (14 days exposure). All sections of the ventral horn of the cervical region of the spinal cord were hybridized with the antisense probe (truncated to 75 nucleotides and at a concentration of 2×10^5 dpm/μl). Dark-field photomicrographs (×45). A. Fixed by perfusion with 4% PA and 0.4% GA followed by proteinase K treatment. B. Fixed by perfusion with 4% PA without proteinase K treatment. C. Fixed by immersion in 4% PA without proteinase K treatment.

The results obtained with the antisense probe in sections of the basal forebrain indicated that decreasing of probe length increased intensity of signals. The highest intensity of signals was observed when the antisense probe was truncated to approximately 75 nucleotides. The probe at a concentration of 2×10^5 dpm/μl was the best in terms of signal-to-noise ratios among concentrations checked.

The results obtained with the sense probe in sections of the neocortex and caudate-putamen exhibited that RNase A treatment after hybridization reduced nonspecific background signals dramatically (Fig. 3).

Hybridization signals were clearly detected after exposure for 7 days, and exposure for 11 days appeared to be optimal in terms of signal-to-noise ratios.

Comparison of ISHH with IHC

For ISHH, tissues were fixed by perfusion with 4% PA and 0.4% GA. Sections were treated with proteinase K (100 μg/ml) at room temperature for 10 min. Probes were truncated to approximately 75 nucleotides and applied to sections at a concentration of 2×10^5 dpm/μl. Autoradiographic exposure was 11 days. Under these optimal conditions, distribution and intensity of signals of labeled neurons in the forebrain, cranial nerve motor

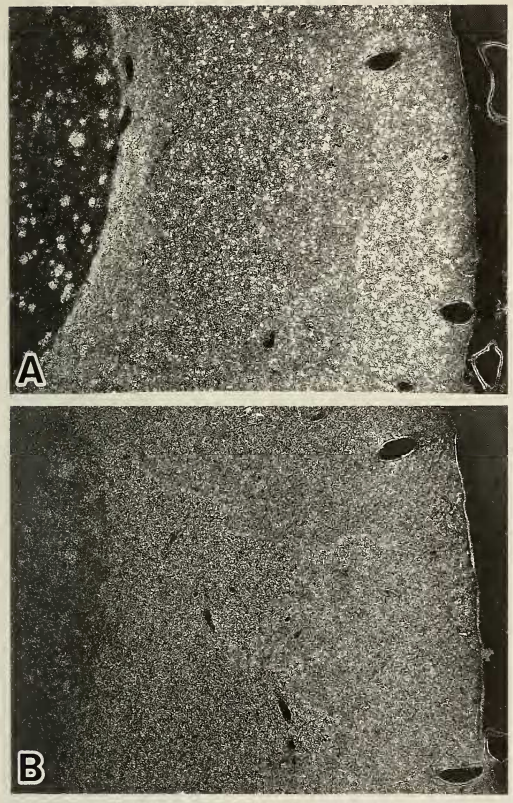

FIG. 3. Effect of RNase A treatment after hybridization on ISHH (11 days exposure). The tissue was fixed with 4% PA and 0.4% GA. Serial sections containing the neocortex and caudate-putamen were hybridized with the sense probe (truncated to 75 nucleotides and at a concentration of 2×10^5 dpm/μl), and untreated (A) or treated (B) with RNase A. Dark-field photomicrographs ($\times$100).

nuclei and cervical region of the spinal cord did not differ appreciably in each region among 6 rats used. On the other hand, distribution of ChAT-immunoreactive neurons in these areas revealed by the present IHC was in agreement with those in previous immunohistochemical studies using monoclonal antibodies to rat ChAT [1, 11, 14, 15]. Immunoreactivity of labeled neurons in each region was consistent among 3 rats used. These observations made it possible to compare distribu-

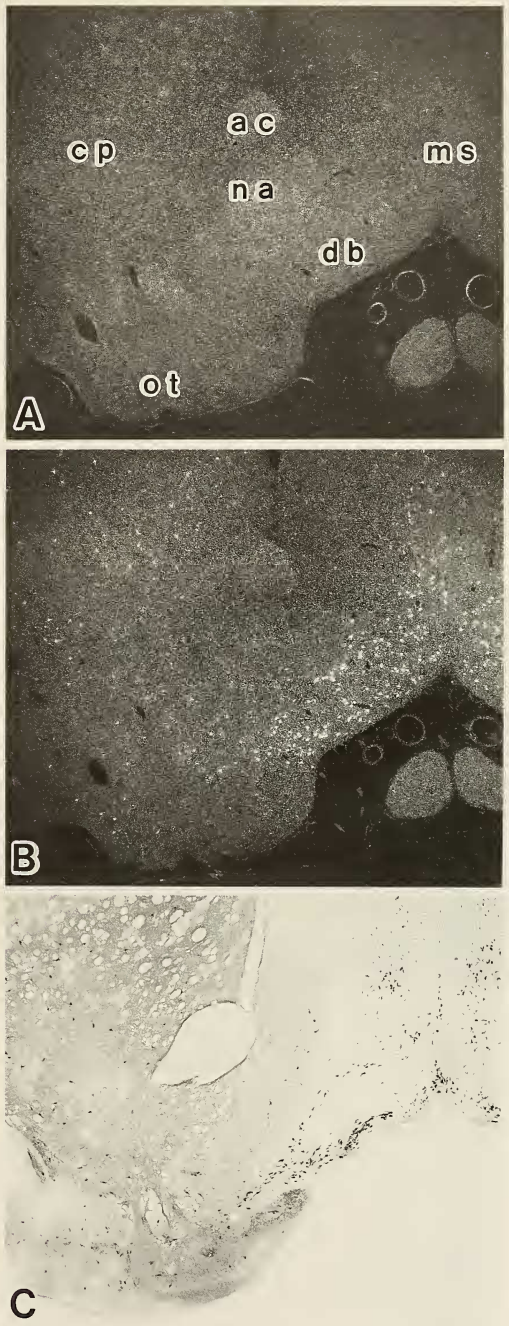

FIG. 4. ISHH and IHC of the basal forebrain. A and B. Dark-field photomicrographs of adjacent 10-μm paraffin sections hybridized with the sense (A) and antisense (B) probes ($\times$20). C. IHC of ChAT in 50-μm frozen section of the similar area to A and B ($\times$20). Note compatible distributions of labeled neurons detected by both methods. No signal was observed in the section hybridized with the sense probe. ac, anterior commisure; cp, caudate-putamen; db, diagonal band of Broca; ms, medial septal nucleus; na, nucleus accumbens; ot, olfactory tubercle.

tion and way of labeling of perikarya of neurons detected by ISHH and IHC in different rats.

In the basal forebrain, labeled neurons by ISHH with the antisense probe were present in the olfactory tubercle (Fig. 4B), medial septal nucleus (Fig. 4B), diagonal band of Broca (Fig. 4B), substantia innominata and magnocellular preoptic nucleus. No signal was observed in the adjacent section hybridized with the sense probe (Fig. 4A). In these areas, distribution pattern of ChAT-immunoreactive neurons was compatible with that of hybridization-positive neurons (Fig. 4B, C). Hybridization signals and immunoreactivity of neurons revealed by the two methods were both intense.

In the neocortex, no cell was detected by ISHH, although weak ChAT-immunoreactive neurons were present in layers II-VI. In the caudate-putamen and nucleus accumbens, neurons detected by both methods showed similar topography (Fig. 4B, C). These neurons were strongly immunoreactive, but showed weak hybridization signals. In the globus pallidus, strongly ChAT-immunoreactive neurons were present, but no hybridization signal was observed. In the medial habenular nucleus, all neurons were positive by

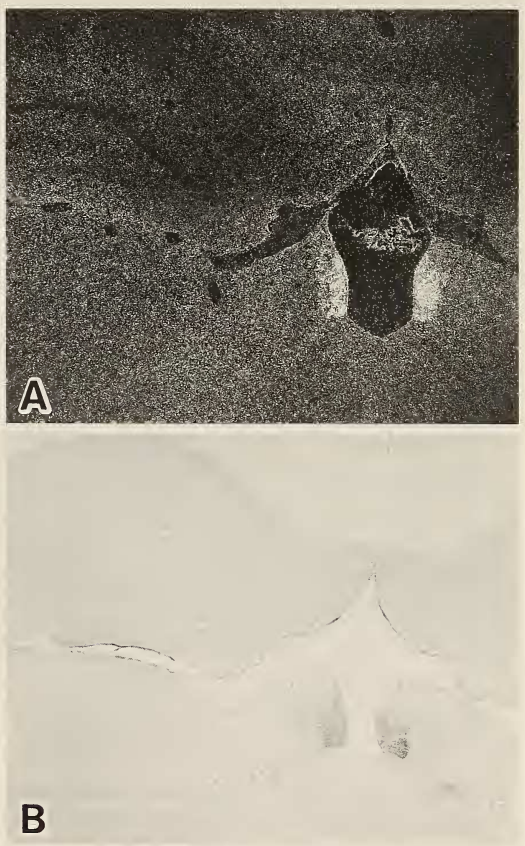

Fig. 5. ISHH and IHC of the medial habenular nucleus. A. Dark-field photomicrograph of the section hybridized with the antisense probe (×20). B. IHC of ChAT (×20).

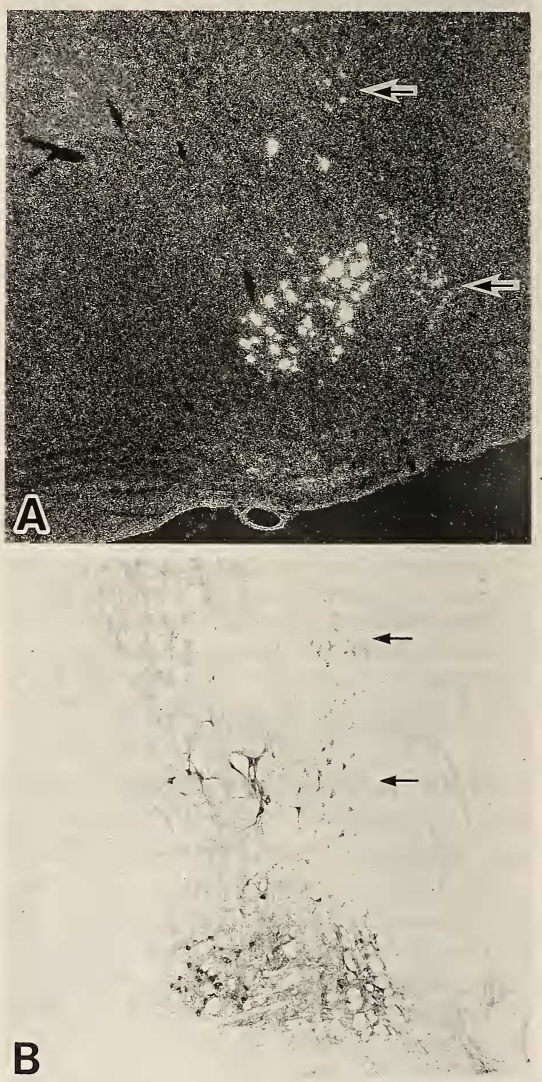

Fig. 6. ISHH and IHC of the motor nucleus of cranial nerve VII. A. Dark-field photomicrograph of the section hybridized with the antisense probe (×30). B. IHC of ChAT (×30). Note that the smaller neurons near the nucleus (arrow) were also detected by both methods.

both methods (Fig. 5). They exhibited intense hybridization signals while their immunoreactivity was weak.

In the brainstem, motoneurons in the motor nuclei of cranial nerves III-VI, VII (Fig. 6), X (Fig. 7) and XII (Fig. 7) were labeled by both methods. Hybridization signals and immunoreactivity of neurons in the motor nuclei of cranial nerves III, IV and VI revealed by both methods were moderate, while those in the motor nuclei of cranial nerves V, VII and XII were intense. In neurons in the motor nucleus of cranial nerve X, hybridization signals were intense, but immunoreactivity was weak (Fig. 7). In the nucleus ambiguus, neurons were strongly immunoreactive, but they showed no hybridization signal (Fig. 7). In the cervical region of the spinal cord, motoneurons were labeled intensely by both methods (Fig. 1). Thus, distribution patterns of perikarya of neurons revealed by ISHH and IHC were almost superimposable, but intensity of hybridization signals did not necessarily correlate with that of immunoreactivity.

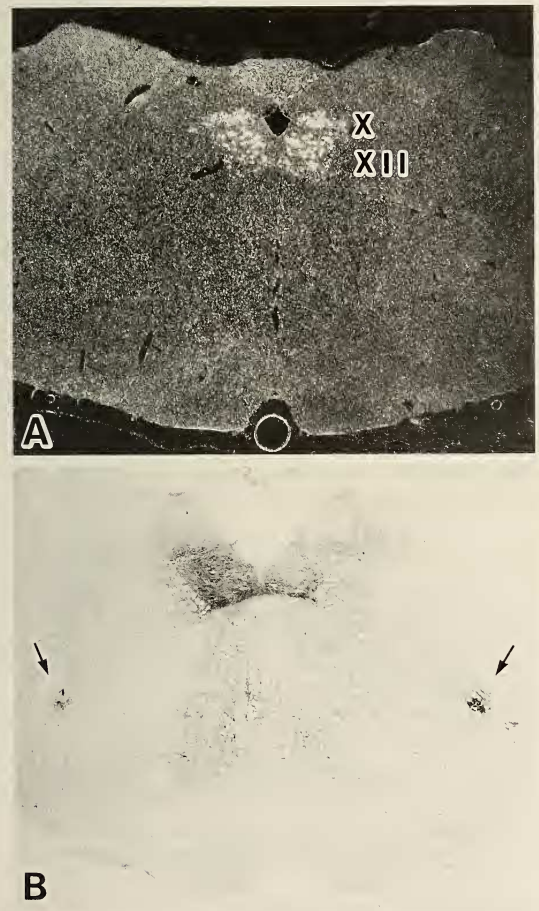

FIG. 7. ISHH and IHC of the motor nuclei of cranial nerves X (X) and XII (XII). A. Dark-field photomicrograph of the section hybridized with the antisense probe (×20). B. IHC of ChAT (×20). Note that neurons in the nucleus ambiguus were not detected by ISHH, but detected by IHC (arrow).

DISCUSSION

ISHH has recently become a widely used procedure for detection and localization of mRNAs in tissue sections [16, 17]. RNA probes have several advantages over other probes as described in Introduction. Cryostat sections are widely used for ISHH and we have experienced that cryostat sections gave slightly higher hybridization signals than paraffin sections. However, some problems inherent to such use have become progressively apparent. One of these is the difficulty in obtaining good details of tissue morphology, as a result of cryostat section thickness and quality. Another major difficulty is the progressive loss of mRNA within sections after prolonged storage [18]. Paraffin sections may be stored for up to 18 months without apparent loss of hybridization signals [19]. In the present study, therefore, we established ISHH for ChAT mRNA in paraffin sections using RNA probes.

Specificity of the present method was confirmed by demonstrating that the antisense probe revealed ChAT transcripts in motoneurons in the spinal cord, while the sense probe exhibited no hybridization signals. In addition, compatible distributions of neurons in most areas in the forebrain and cranial nerve motor nuclei revealed by ISHH and IHC indicated that the present method was highly specific. So far, there are two reports on ISHH for ChAT mRNA using ^{35}S-labeled oligonucleotide probes in cryostat sections of the forebrain of the rat [20] and of the brainstem of the rat and guinea pig [21]. We demonstrated that use of full length of the RNA probe, which was truncated to approximately 75 nucleotides after labeling, gave much higher intensity of signals and lower

background signals than use of the oligonucleotide probe. Higher intensity of signals may be due to higher specific activity of the RNA probe.

Efficiency of the present ISHH depended on the fixation procedure. Fixation by perfusion with 4% PA and 0.4% GA in combination with proteinase K treatment appeared to be the most favorable fixative. Using this protocol, we have succeeded in detecting mRNA for Ca^{2+}/calmodulin-dependent protein kinase II in the rat brain (T. Ichikawa, S. Ohsako and T. Yamauchi, unpublished). We have also detected mRNA for hydroxyindole O-methyltransferase in the bovine epithalamus fixed by immersion in 4% PA and 0.1% GA in combination with proteinase K treatment [22]. It should be noted, however, that mRNA for arylamine N-acetyltransferase in the chicken kidney was detected by our protocol only when the tissue was fixed by immersion in 4% PA [23], indicating that the appropriate fixation procedure might differ among tissues used.

Considerable loss of sections or parts of sections from slides during the hybridization procedure was encountered using slides coated by a variety of procedures, including gelatin [24], egg white, "Histostik" [25] or polylysine [26]. Using slides coated with 1% BSA and fixed with 25% GA, we have overcome this problem.

Probes whose mass average size is about 150 nucleotides are empirically used [8]. In the present study, more intense signals were observed when the probe was truncated to approximately 75 nucleotides. However, further truncation might cause a significant reduction in hybrid melting temperature (Tm), or much variation in Tm as a function of difference in fragment length. In addition, we have experienced that further truncation decreased specificity and stability of probes.

Intensity of hybridization signals for ChAT mRNA correlated with immunoreactivity for ChAT in neurons in the basal forebrain, motor nuclei of cranial nerves III-VII and XII and spinal cord. In contrast, neurons in the medial habenular nucleus and motor nucleus of cranial nerve X exhibited intense hybridization signals but weak immunoreactivity, while neurons in the caudate-putamen and nucleus accumbens showed weak hybridization signals but strong immunoreactivity. In addition, neurons in the neocortex, globus pallidus and nucleus ambiguus were not detected by ISHH, although ChAT-immunoreactive neurons were present there. Of necessity, comparison of intensity of hybridization signals and immunoreactivity in neurons detected by ISHH and IHC was performed in different rats in the present study. The absence of a constant correlation between hybridization signal abundance and immunoreactivity in the same neuron has been reported [27–29]. Discrepancies between amounts of mRNA and cellular contents of its translated protein could be due to a number of factors. Prominent among these is presence of endogenous factors that control translation or posttranslational events [30, 31]. Other possibilities include the relative sensitivities of ISHH and IHC. Amounts of mRNA in neurons in the neocortex, globus pallidus and nucleus ambiguus may be below the sensitivity of the present ISHH.

Establishment of ISHH for ChAT mRNA in paraffin sections may be of particular use to study the embryonic and early postnatal development of cholinergic neurons. Because the time or site of synthesis of mRNA may differ from the time or site of accumulation of its translated protein [32], studies using ISHH in combination with IHC may provide more precise information on the developing cholinergic neurons.

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